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Bacterial Endosymbionts in the Gills of the Deep-Sea Wood-Boring Bivalves *Xylophaga atlantica* and *Xylophaga washingtona*

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Abstract. Bacterial endosymbionts found in gill tissues in several bivalve families convert otherwise unavailable energy sources (sulfide, methane, or cellulose) to forms readily metabolized by their hosts. We investigated the existence of such a symbiosis in two species of *Xylophaga* (family Pholadidae). The genus *Xylophaga* includes opportunistic species that are the predominant colonizers of wood at depths greater than 150 m. It has been hypothesized that, like their shallow-water counterparts the shipworms (family Teredinidae), species of *Xylophaga* utilize wood for nutrition. Results from transmission and scanning electron microscopy of *X. atlantica* and *X. washingtona* clearly demonstrate the presence of endosymbionts that resemble the shipworm endosymbionts both morphologically and in their anatomical location within the gills. *Xylophaga* and the teredinids both have a caecum packed with wood chips but lack the dense populations of microorganisms associated with cellulose digestion in termites or ruminants. These observations suggest that *Xylophaga* has evolved a symbiotic solution to wood digestion similar to that seen in shipworms. Hence, the *Xylophaga* symbiosis suggests a mechanism for the conversion of terrestrially derived cellulosic carbon from wood into animal biomass in the deep sea.

Introduction

In 1973 Popham and Dixon (Popham and Dickson, 1973) demonstrated the presence of endosymbiotic bacteria in the gills of wood-boring bivalves of the family Teredinidae, commonly known as shipworms. The role of these symbionts, however, was not elucidated until a decade later when it was shown that the symbiotic bacteria, when cultivated *in vitro*, both digest cellulose and fix atmospheric nitrogen (Waterbury *et al.*, 1983). This novel combination of symbiont metabolic activities is now thought to be essential for the survival of teredinids on a diet composed primarily of wood, a food source that, although rich in carbon and energy, is deficient in nitrogen and cannot be digested by most animals.

Whereas teredinids are the predominant wood-boring bivalves in shallow waters (intertidal to 100 m), bivalves of the subfamily Xylophagainae (family Pholadidae) fill the same niche in the deep oceans, occurring primarily at depths from 150 m to greater than 7000 m (Turner, 1972). Like teredinids, xylophagainids bore into woody substrates, ingest the excavated wood particles, and store them in a specialized caecum formed by an outpocketing of the stomach (Purchon, 1941; Turner, 1973). The role of wood in the diet of *Xylophaga* has not, however, been determined experimentally. On the basis of morphological and ecological arguments, it has been proposed that Xylophagainids derive (1) significant (Purchon, 1941; Turner, 1973), (2) little (Potts, 1923), or (3) no (Yonge, 1937, 1938; Yonge and Thompson, 1976) nutrition from the ingestion of wood.

The utilization of wood by xylophagainids is a subject of considerable ecological interest since wood is abundant in many areas of the deep sea (Wolff, 1979) and the opportunistic Xylophagainae are among the most common colonizers of wood at these depths (Turner, 1973). Hence the deep-sea Xylophagainae could perform a role analogous to that of termites in terrestrial habitats and shipworms in shallow marine habitats by converting the refractory cellulosic carbon deposited in wood and other plant remains to a more readily available form (Turner,

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1973). Both termites (Kane, 1997) and shipworms (Waterbury *et al.*, 1983) utilize cellulolytic and nitrogen-fixing symbionts to survive on wood as the sole food source. In this report we present evidence that bacterial endosymbionts are present in the gill tissue of two species of *Xylophaga*, and that these bacteria appear morphologically similar to the gill endosymbionts previously identified in shipworms.

Materials and Methods

Specimens of *X. washingtona* were collected in pine boards submerged for 2–3 months in Monterey Bay (depth 61 m, coordinates 36°39.8′ N, 121°52.88′ W; courtesy of Dr. E. C. Haderlie, Naval Postgraduate School) or in Scripps canyon off the San Diego coast (depth 274 m, coordinates 32°31′ N, 117°16.5′ W). Specimens of *X. atlantica* were collected from oak boards (1″ × 2″ × 2′ lobster-trap skids) submerged for about 1 year at 80–100 m depth 12 miles off the coast of SW Harbor, Maine. Animals were kept alive in chilled seawater tanks until they were removed from the wood and prepared for microscopy (less than 2 weeks). Animals with an average valve diameter of 3–4 mm were dissected and fixed for 1–1.5 h in 3% glutaraldehyde buffered with 0.1 *M* cacodylate/HCl (pH 7.3) and 0.4 *M* NaCl or 3% glutaraldehyde buffered with 0.1 *M* sodium phosphate (pH 7.4), 3% NaCl, and 4.5% sucrose as in Eckelbarger *et al.* (1990). After fixation the specimens were post-fixed in 1% osmium tetroxide and dehydrated through a graded ethanol series. Specimens for transmission electron microscopy (TEM) were then transferred to propylene oxide and embedded in Epon/Araldite for sectioning. Specimens for scanning electron microscopy (SEM) were fractured under liquid nitrogen after ethanol dehydration and then were critical-point dried from CO_2 and sputter coated with gold. A Phillips CM10 transmission electron microscope and an AMR 1000 scanning electron microscope were used to examine samples.

Both *Xylophaga atlantica* and *Xylophaga washingtona* were used for TEM of the gills and light microscopy of the digestive tract. Similar results were observed with both species. *X. atlantica* was used for SEM of the gills, and *X. washingtona* was used for TEM of the digestive tract.

Results

Gross morphology of the ctenidium

The gross morphology of the gills of *Xylophaga* has been described in detail by Purchon (1941) and will be briefly summarized here. The ctenidia (gills) of Xylophagainae, like those of the Teredinidae, are formed by a single (outer) demibranch on either side of the visceral mass (Fig. 1). The marginal groove is absent, there is no ciliary sorting mechanism, and the labial palps are greatly reduced. These features are shared by most tere-

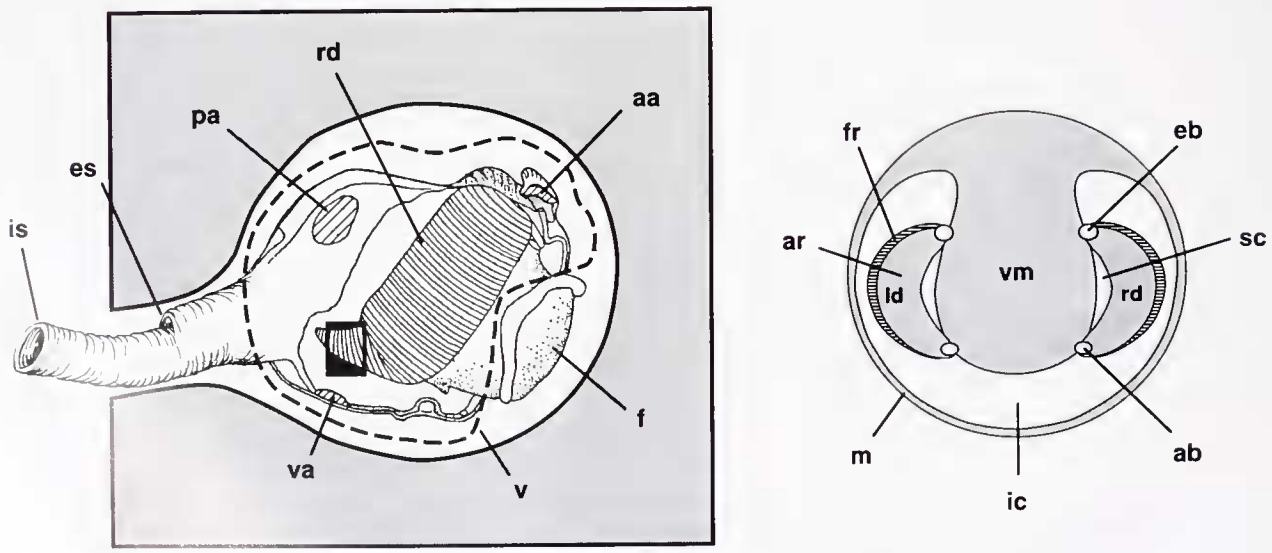

Figure 1. Diagram of *Xylophaga* in sagittal section shown in the burrow (left) and in transverse section (right). aa, anterior adductor muscle; ab, afferent branchial vein; ar, abfrontal region of gill demibranch; eb, efferent branchial vein; es, exhalent siphon; f, foot; fr, frontal region of gill demibranch; ic, infrabranchial chamber of mantle cavity; is, inhalant siphon; ld, left demibranch of gill; m, mantle; pa, posterior adductor muscle; rd, right demibranch of gill; sc, suprabranchial chamber of mantle cavity; va, ventral adductor muscle; v, outline of valves. Boxed area is shown in Figure 2C.

dinids but are divergent from the filter-feeding gill morphology of other lamellibranchs (Purchon, 1941). The ctenidia are connected to the visceral mass dorsally at the efferent branchial vein and ventrally at the afferent branchial vein, forming an arc that separates the mantle cavity into suprabranchial and infrabranchial chambers. Each filament is composed of two distinct regions: a narrow frontal region (fr) and a broad, flattened abfrontal region (ar). As is typical of lamellibranch gills, the frontal region is composed of frontal, laterofrontal, and lateral ciliated cells; a central blood vessel; and supporting connective tissue. The abfrontal region, however, is distinctive, consisting of an extensively developed interlamellar junction that forms a crescent-like sheet bridging the arc of the filament (Fig. 1). The filaments are joined laterally by interfilamentar cellular junctions in the frontal region. Whereas cellular interfilamentar junctions are absent in the abfrontal region, filaments appear to be connected by lateral ciliary junctions in this region (Fig. 2E).

Identification and localization of intracellular bacteria in the ctenidium

In the Xylophagainae, as in other symbiont-bearing bivalves, the frontal region of each gill filament is devoid of symbionts. The most conspicuous cytoplasmic components of the cells of the frontal region include mitochondria, Golgi, endoplasmic reticulum, ciliary structures, centrioles, and glycogen granules. Bacterial symbionts are, however, abundant in the abfrontal region of the ctenidium (Fig. 2D–F and Fig. 3). This region forms a broad, flattened plate composed of two closely appressed single-layered sheets of epithelial cells surrounding a narrow blood sinus.

The epithelium of the abfrontal region consists of two recognizable cell types here referred to as *bacteriocytes* and *intercalary cells* in keeping with terminology in current use for other bivalve symbioses (Fisher, 1990; Frenkiel *et al.*, 1996; Gros *et al.*, 1996). Bacterial endosymbionts are absent from the intercalary cells but dominate the cytoplasm of the bacteriocytes. The symbionts are distributed in clusters throughout the bacteriocytes, with mitochondria infrequently interspersed in the cytoplasm between clusters. Large membrane-bound inclusions, possibly lysosomes or lysosomal residual bodies, also occur in the bacteriocyte cytoplasm (Fig. 3). These inclusions frequently display a biphasic appearance with a granular low-density region and a slightly denser reticulate region that appears to consist of whorled membranes. Some inclusions contain bodies suggestive of partially degraded bacteria. Bacteriocytes and intercalary cells are uniformly distributed in the epithelium of the abfrontal region with about equal frequency. The apical surfaces of both cell types are densely covered with microvilli at points that directly contact the external environment (Fig. 3C).

The bacteriocytes are typically spherical or cylindrical. Their broad basal surfaces compose much of the internal lining of the blood sinus. Intercalary cells, on the other hand, are typically narrow at their basal ends, expanding to a broad and irregular apical surface. Thin sheet-like projections of the apical end of the intercalary cells extend over the apical surfaces of adjacent bacteriocytes, apparently shielding most but not all of the surface of the bacteriocytes from direct contact with the external environment. The outer surface of the abfrontal epithelium is elaborated into a series of larger rounded hummocks (formed by the spherical outline of the bacteriocytes) and smaller papillae (formed by the irregular surfaces of intercalary cells) surrounded by many deep folds and pits (Fig. 2F and Fig. 3A). The inner blood-facing surface of this epithelium also contains numerous invaginations (Fig. 3). These elaborations greatly increase the surface areas that are exposed to blood internally and to seawater externally in the symbiont-containing abfrontal zone.

The endosymbionts are straight to gently curved rods that display a cell-wall morphology typical of gram-negative bacteria and range from 0.4 to 0.7 μm in width and up to 5.0 μm in length (Fig. 3). No conspicuous internal structures, such as the sulfur granules seen in thiotrophic symbionts or the stacked internal membranes seen in methanotrophic symbionts of other bivalve families (Fisher, 1990), are observed in the symbionts of *Xylophaga*.

The symbionts are not in direct contact with the cytoplasm but are contained within membrane-bound vesicles ranging from 10 to 20 μm in diameter. A single vesicle may contain many bacteria. The orientation of symbionts within vesicles is not random. Most symbionts are oriented with their long axes perpendicular to the lateral surface of the filament. Few symbionts are oriented with their long axes parallel to the lateral surface and of those, fewer still align with the frontal-abfrontal axis of the filament. This distribution of orientations is readily observable in both SEM and TEM images.

Extensive examination of TEM images failed to provide evidence of connections between the symbiont-containing vesicles and the external environment. In all sections examined, the vesicular membrane appears distinct from and unconnected to the plasma membrane. Similarly, examination of the exterior surfaces by SEM revealed no evidence of the kind of ducts or openings to the bacteria-containing vesicles that are seen in the symbiont-containing light organs of some luminous fish (Haygood, 1993). Although deep pits and infoldings were observed on the lateral surfaces of the filaments in SEM images, TEM images showed that these are not

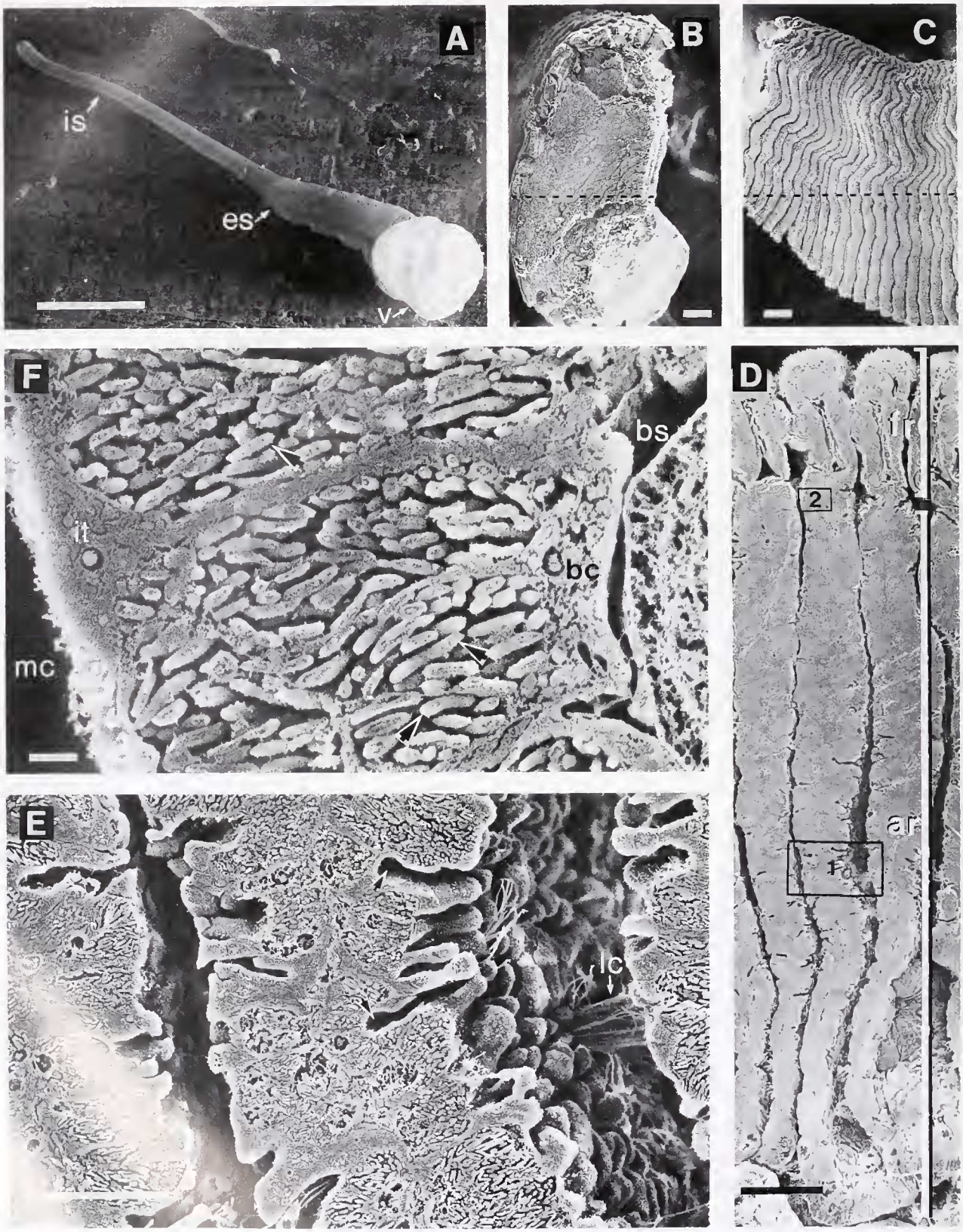

Figure 2. (A) *Xylophaga washingtona* with siphon fully extended. (B–F) Scanning electron micrographs of *X. atlantica* gill. (B) Lateral surface of a gill filament near the posterior tip of the right demibranch. (C) Frontal surface of the right demibranch. (D) Transverse fracture of right demibranch showing three gill

continuous with the symbiont-containing vesicles (Fig. 3C). Hence, at minimum, the bacterial symbionts are separated from the external environment by the plasma membrane, cytoplasm, and vesicular membrane of the host.

Wood and flora of the digestive tract

Xylophaga has a digestive system similar to that described for shipworms, including a large caecum that is typically distended with wood shavings (Fig. 4). The dimensions of the shavings (width and height) match the contours of the dentition on the shell, consistent with the ingestion of wood excavated during burrowing. Wood was the predominant component of the gut contents identifiable at the level of light microscopy. The caecum was the most densely packed space, while the stomach contained dispersed wood fibers. Fibrous material was found in proximity with the crystalline style and at low density in the style sac. Examination of serial thick sections of the guts of several animals failed to reveal any conspicuous community of microorganisms resembling those found in wood-eating insects. Electron microscopy confirmed this observation and further indicated that bacteria are absent from cells of the ciliated epithelium lining the gut and occur only sparsely in the digestive spaces (Fig. 4D–E).

Discussion

The discovery of bacterial endosymbiont populations in a number of marine invertebrate species has been the key to understanding the remarkable ability of these invertebrates to survive on unusual nutrient sources. For example, the identification of cellulolytic, nitrogen-fixing bacteria in the gills of the wood-boring teredinid clams (shipworms) pointed toward an explanation of how these bivalves are able to thrive with wood as their primary food source. This capability, which is quite rare among higher animals, has led to the great success of the Teredinidae as colonizers of wood in coastal marine environments. In the deep oceans, the dominant colonizers of wood and other plant materials are the Xylophagainae (family Pholadidae). Similarities in the gut anatomy and wood-boring habits of the Teredinidae and the Xylophagainae led us to examine whether species of *Xylophaga* maintain similar symbiotic associations. In this report we present morphological evidence for the existence of bacterial endosymbionts in the gill tissue of two of these deep-sea wood-boring bivalves—*Xylophaga atlantica* and *Xylophaga washingtona*.

In many respects, the gill of *Xylophaga* closely resembles the symbiont-containing gills of other bivalves including the Lucinidae, Vesicomyidae, Modiolinae, and Solemyidae (Distel and Felbeck, 1987; Fisher 1990; Frenkiel *et al.*, 1996; Gros *et al.*, 1996). The gill of *Xylophaga* is composed of two regions: a heavily ciliated, symbiont-free frontal region that is like the typical lamellibranch gill structure, and an abfrontal region that appears to be specifically adapted to harbor symbiotic bacteria. The abfrontal region, an extension of the interfilamentar junction, is much broader than the frontal region and composes the bulk of the mass of the gill. The symbionts are completely surrounded by a vacuolar membrane of host origin and lack contact with the external environment. In addition to harboring symbionts, the bacteriocytes contain large membranous structures resembling lysosomes in various stages of development. In some instances, these lysosome-like compartments contain bodies that resemble partially degraded bacteria, which suggests that lysosomal digestion is a mechanism for regulation of the bacterial population.

The symbiont-containing tissue in *Xylophaga* also resembles that of the teredinids. Although teredinid gills are highly modified, the symbionts are found in an anatomically analogous region (Distel *et al.*, 1991). This region is referred to as the gland of Deshayes (Sigerfoos, 1908) in teredinids and corresponds to the interlamellar junction of the fused right and left demibranchs (Turner, 1966; Distel *et al.*, 1991). Bacteriocytes in this tissue contain symbionts that resemble the symbionts of *Xylophaga;* both are gram-negative rods averaging about 0.5 μm in width and up to 5 μm in length, and both lack internal membranes or other conspicuous internal or external structures.

It is striking that xylophagainids and teredinids not only harbor symbionts that are similar in appearance and location, but that they also share similar adaptations to their woody habitats. Both are obligatory wood borers that excavate burrows by using their shells as rasps, ingest the excavated wood shavings, and store the wood parti-

filaments (plane of fracture indicated by dashed lines in B and C). Abfrontal (ar) and frontal (fr) regions indicated by side bar. (E) Detail from box 1 in D, showing deep invaginations (arrows), lateral ciliary tufts (lc), and numerous papillae decorating the lateral surfaces of the abfrontal region. (F) Detail from box 2 in D, showing an intercalary cell (il) and portions of two bacteriocytes (bc). Rod-shaped cells (arrows) within bacteriocytes are symbionts. mc, lumen of mantle cavity; bs, blood sinus; es, exhalent siphon; is, inhalant siphon; v, valve. Scale bars: A, 0.5 cm; B–C, 100 μm; D, 50 μm; E, 10 μm; F, 1.0 μm.

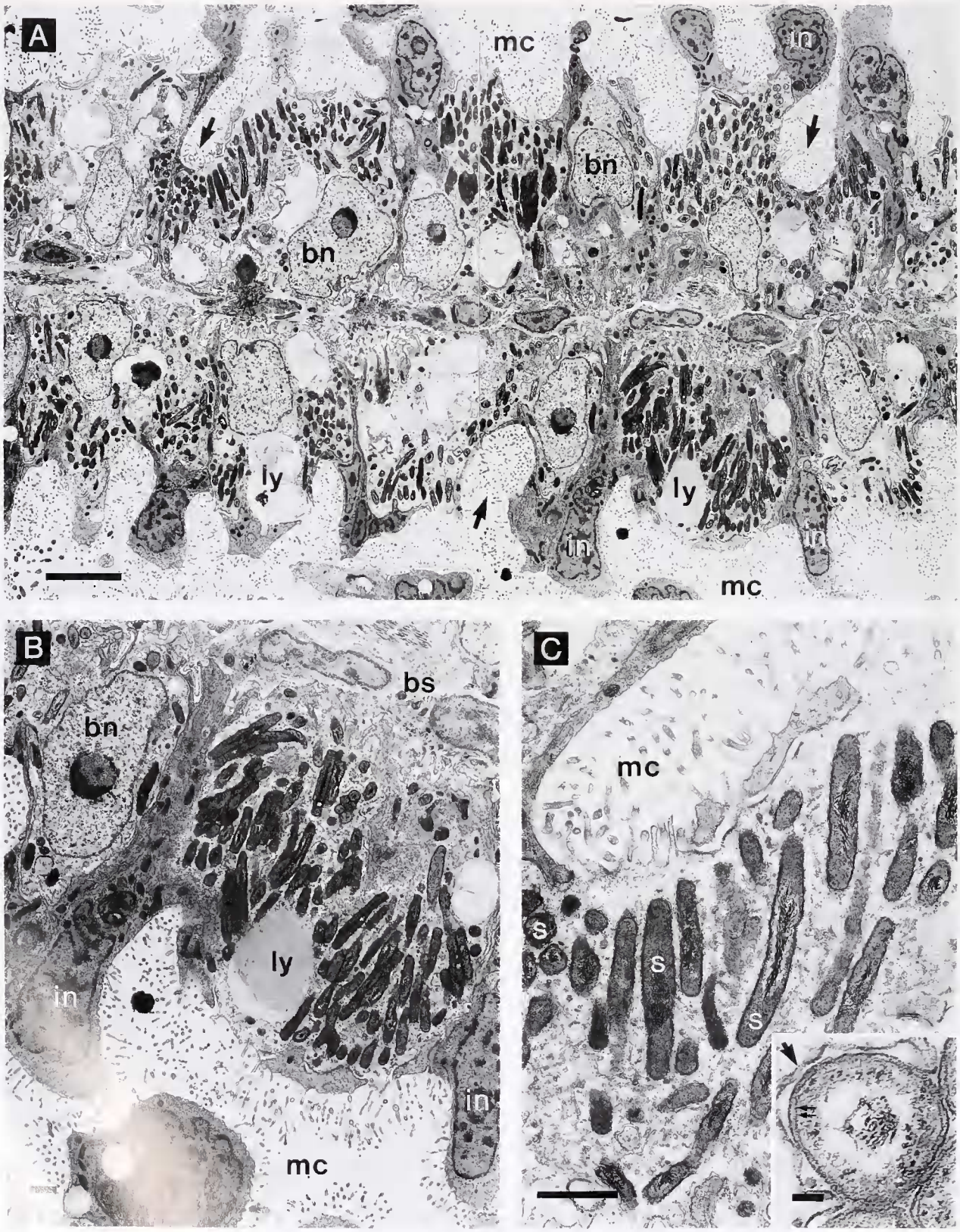

Figure 3. Transmission electron micrographs of a section through the abfrontal region of the gill of *Xylophaga atlantica*. (A) Low-magnification view of a region comparable to that shown in 2E. Symbionts appear as electron-dense rods. Note deep invaginations of the microvillar surface of gill (arrows). (B) Detail

cles in a large caecum prior to passing them through the stomach and gut. Purchon (1941) argued that several features common to the xylophagainid and teredinid digestive systems indicate a departure from reliance on filter feeding and represent specific adaptations to the digestion of wood. These include the lack of a marginal food groove, limited ciliary sorting mechanisms in the ctenidia, reduced labial palps and crystalline style sac, and presence of a wood-storing caecum with ciliated grooves capable of providing a steady stream of wood particles to the stomach.

It is now well established that wood is a primary constituent of the teredinid diet (Gallager *et al.*, 1981), although nutrients may also be obtained from suspension feeding (Mann and Scott, 1985). In fact, at least one shipworm species has been shown to be capable of sustaining normal growth and reproduction with wood as the sole source of particulate nutrients (Gallager *et al.*, 1981). The shipworm symbiont has been cultivated *in vitro* and shown to fix atmospheric nitrogen and secrete cellulolytic, xylanolytic, and proteolytic enzymes (Greene and Freer, 1986; Greene *et al.*, 1988; Greene, 1989, Greene and De Wispeleare, pers. comm.). Although these symbiont activities are appropriate for a wood-based diet and are thought to be critical for the shipworm's success in colonizing woody substrates, no direct evidence exists for their participation in the nutrition of the shipworm hosts.

Since the *Xylophaga* symbiont has not yet been cultivated, the question of whether its metabolic capabilities are similar to those of the shipworm symbiont is unresolved. Hence, it is possible that the two bacteria play different roles in their respective symbioses. For example, the bacterial symbionts in *Xylophaga* may be nitrogen fixing but not cellulolytic, or they may contribute essential nutrients that are absent from wood. On the other hand, phylogenetic analyses based on 16S rRNA sequences indicate that the *Xylophaga* symbiont is the closest relative of the shipworm isolates identified to date (Distel and Roberts, 1996). That finding increases the likelihood that these two symbionts play similar physiological roles.

Although the presence of similar bacteria in the same regions of the gills of the Teredinidae and the Xylophagainae does not demonstrate a common function for the two symbioses the additional features they have in common—their wood-boring habits and parallel anatomical modifications of the digestive tract—lend support to this hypothesis. To our knowledge, no animal species has yet been demonstrated to utilize wood as a primary nutrient source without the aid of symbiotic microorganisms; thus if wood is the primary dietary constituent of *Xylophaga*, a nutritional role for its symbionts is strongly implicated. If wood is not a primary food source for the xylophagainids (as it is for teredinids), it is difficult to explain the function of the caecum, account for the fact that wood is the principal constituent of the caecal and gut contents, or identify the diet of the xylophagainids. The scarcity of alternative food sources in the deep sea, as well as the notable paucity of bacteria, phytoplankton, and other microorganisms in the gut of *Xylophaga*, seems to preclude the possibilities that *Xylophaga* subsists by filter feeding, by grazing on wood-associated microorganisms, or by utilizing an extracellular symbiotic gut microfauna as do termites and ruminants. Nonetheless, the possibility cannot be ruled out that *Xylophaga* (or the teredinids) produces sufficiently active endogenous cellulases to facilitate wood digestion, as has been suggested for some termites (Breznak and Brune, 1994).

To determine whether the *Xylophaga* symbiosis is cellulolytic, it is necessary to identify the activity of cellulolytic or nitrogen-fixing enzymes in the symbionts, demonstrate the depletion of cellulose in fecal material, and analyze the nutritional utilization of carbon derived from cellulose. These properties have been demonstrated for the shipworm symbiosis, although it is still not clear how the cellulolytic enzymes produced by bacterial endosymbionts in the gill might be transported to the gut where wood digestion must occur. In the teredinids, it has been proposed that a duct in the afferent branchial vein connects the gills to the esophagus (Saraswathy, 1971). Such a duct could serve as a conduit for cellulolytic enzymes; however, the complete vessel was not observed in all species examined, and its existence has not been independently confirmed. Our results indicate that the symbionts of *Xylophaga* (this study) and those of teredinids (unpubl. obs.; Trytek and Allen

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from lower right quadrant of A, showing a bacteriocyte and portions of two neighboring intercalary cells. (C) Detail from upper left quadrant of A, showing symbionts near the base of a deep lateral invagination. Note that membranes surrounding symbionts are distinct from the microvillar surface of the bacteriocyte plasma membrane. Inset in C shows high magnification of a symbiont cell from *X. washingtona*. Large arrow indicates host-derived membrane of symbiont-containing vesicle. Small arrows indicate double-layered gram-negative cell envelope of the symbiont cell. bs, blood sinus; bn, bacteriocyte nucleus; in, intercalary cell nucleus; ly, lysosome-like granules; mc, lumen of mantle cavity; s, symbionts. Scale bars: A, 5 μm, B–C, 1.0 μm; inset, 0.1 μm.

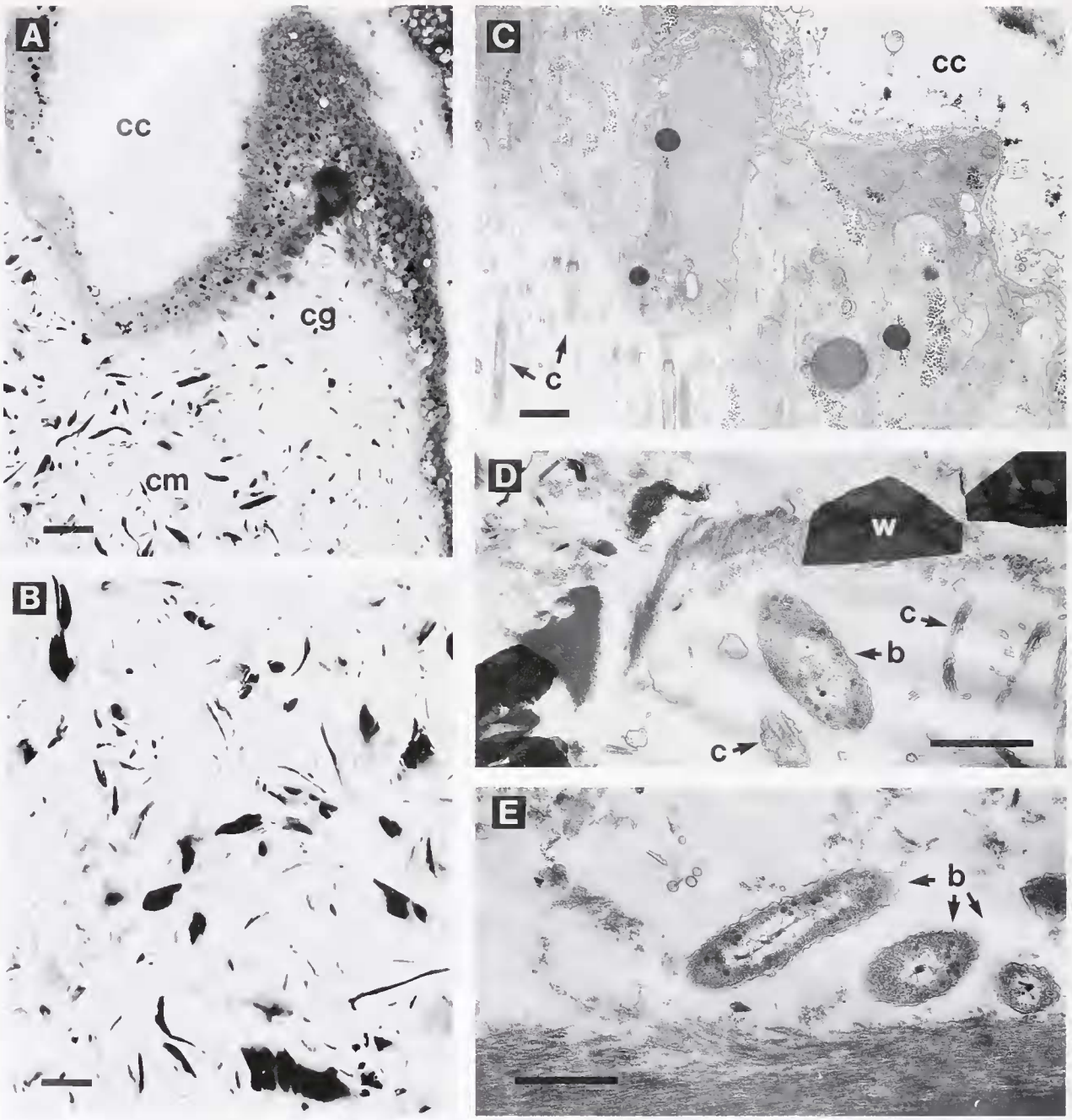

Figure 4. Gut content of *Xylophaga washingtona* (A) Light micrographs of caecal contents showing ciliated groove (cg) that channels wood shavings into the stomach. (B) Detail of caecal contents from A. Note predominance of wood shavings and absence of algae, fungi, and protozoans. (C–E) Transmission electron micrographs. (C) Ciliated epithelial cells in the region of the caecal wall (ciliated groove) shown in A. The cilia in the lower left face into the lumen of the caecum. No bacteria were observed within the cells of the gut epithelium. (D) One of the rare bacteria seen among the caecal contents. The dark, electron-dense pentagons are wood shavings. (E) Cluster of bacteria observed adjacent to the gastric shield of the stomach. b, bacteria; c, cilia; cc, coelomic cavity; cm, caecum; w, wood. Scale bars: A, 250 μm; B, 150 μm; C–E, 1.0 μm.

(1980) truly intracellular. Consequently, the plausibility of a symbiotic mechanism depends on the demonstration of a pathway whereby enzymes from an intracellular bacterial population are ultimately transported to the lumen of the gut to participate in cellulose degradation.

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